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<p>(54) Title: <b>GENE AND PROTEIN INVOLVED IN LIVER REGENERATION</b></p> <p>(57) Abstract</p> <p>The present invention relates to a gene involved in regeneration processes of the liver and comprising a nucleotide sequence which is at least 70 % homologous to the sequence of figure 1 or the sequence of figure 6, or the complementary strand thereof, for use in the design of PCR probes for detecting nucleotide sequences in a source material, which nucleotide sequences represent genes corresponding with the gene sequence of figure 1 or the sequence of figure 6; protein encoded by said gene for use in diagnosis of liver regeneration and/or liver cell proliferation; and antibodies directed against this protein, a PCR primer comprising at least part of said gene as a probe, a single stranded nucleotide sequence being at least in part complementary to the messenger RNA transcribed from said gene as a probe an expression vector and a host cell comprising said nucleotide sequence, for use in a method for detecting the occurrence of liver cell proliferation in a subject.</p>																																							
<p><b>Expression of rat liver RAP3 mRNA (large size)</b></p> <table border="1"> <caption>Data for Expression of rat liver RAP3 mRNA (large size)</caption> <thead> <tr> <th>Time (hours)</th> <th>sham operation (units)</th> <th>70% hepatectomy (units)</th> </tr> </thead> <tbody> <tr> <td>8</td> <td>~4500</td> <td>~22000</td> </tr> <tr> <td>12</td> <td>~4500</td> <td>~6500</td> </tr> <tr> <td>18</td> <td>~3500</td> <td>~2500</td> </tr> <tr> <td>24</td> <td>~7500</td> <td>~2000</td> </tr> <tr> <td>30</td> <td>~5000</td> <td>~2500</td> </tr> </tbody> </table> <p><b>Expression of rat liver RAP3 mRNA (small size)</b></p> <table border="1"> <caption>Data for Expression of rat liver RAP3 mRNA (small size)</caption> <thead> <tr> <th>Time (hours)</th> <th>sham operation (units)</th> <th>70% hepatectomy (units)</th> </tr> </thead> <tbody> <tr> <td>6</td> <td>~2500</td> <td>~7500</td> </tr> <tr> <td>12</td> <td>~4000</td> <td>~5500</td> </tr> <tr> <td>18</td> <td>~3000</td> <td>~4000</td> </tr> <tr> <td>24</td> <td>~1500</td> <td>~3500</td> </tr> <tr> <td>30</td> <td>~4000</td> <td>~2500</td> </tr> </tbody> </table>				Time (hours)	sham operation (units)	70% hepatectomy (units)	8	~4500	~22000	12	~4500	~6500	18	~3500	~2500	24	~7500	~2000	30	~5000	~2500	Time (hours)	sham operation (units)	70% hepatectomy (units)	6	~2500	~7500	12	~4000	~5500	18	~3000	~4000	24	~1500	~3500	30	~4000	~2500
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## GENE AND PROTEIN INVOLVED IN LIVER REGENERATION

The present invention relates to the detection of a novel gene and protein involved in liver cell proliferation. The gene and protein and related molecules, such as nucleotide probes derived from the 5 gene and antibodies directed to the protein form also part of the invention. The gene will be identified herein as RAP3 gene. The corresponding protein is called RAP3 protein.

The adult liver has the capacity to regenerate 10 after damage or partial resection. This process may allow for recovery from hepatic injuries caused by viruses, toxins, ischemia, surgery, and auxiliary liver transplantation. Liver regeneration has been studied extensively in the rat after a 70% partial hepatectomy. 15 During the first four hours following partial hepatectomy there is a rapid, transient transcriptional activation of genes involved in the immediate early response. After induction of these immediate early genes during the transition from the quiescent state of the liver ( $G_0$ ) to 20 the growth phase ( $G_1$ ), a delayed early gene activation is initiated which peaks during the transition of the  $G_1$  to the DNA synthesis phase (S phase).

In the research that led to the present invention novel genes involved in the delayed early 25 response were identified by analyzing gene expression in rat liver at six hours after 70% partial hepatectomy. Upregulated genes were selected by cDNA subtractive hybridization. Upregulation was quantified by Northern blotting and the truly upregulated genes were 30 characterized by sequence analysis.

Twelve genes were found to be upregulated at different degrees (1.5 to 10.4 fold) six hours after partial hepatectomy. Sequence analysis revealed that eight of the upregulated genes have previously been 35 reported to be associated with liver regeneration or cell proliferation in general, one has previously been assigned an unrelated function and three have no sequence similarity to known genes.

The various upregulated genes showed two distinct gene expression patterns during a 30 hour period after partial hepatectomy. The first pattern has two peaks coincident with the G<sub>1</sub> phases of two consecutive 5 hepatic cell cycles. The second one shows a narrow peak at six hours after which the gene is downregulated. The novel RAP3 gene (RAP: regeneration associated protein) which was most upregulated (3.3 fold), showed the latter gene expression pattern.

10 The full length cDNA of this gene was isolated from a rat liver cDNA library. Sequence analysis showed two full length cDNAs of 1282 and 1834 bp, respectively, encoding a novel protein of 367 amino acid residues. Figures 1A and 1B show the nucleotide sequence of the 15 cDNA's. Figure 2 shows the derived amino acid sequence.

In addition, the cDNA of the human RAP3 gene was isolated. Sequence analysis showed two cDNAs of 1282 and 1867 bp respectively, encoding a protein of 363 amino acids. Figures 6A and 6B show the nucleotide sequence of 20 the cDNA's. Figure 7 shows the derived amino acid sequence. The 1867 human RAP3 cDNA shows a 76% homology with the 1834 rat RAP3 cDNA.

On the basis of this finding it became possible to design probes, primers and reagents for use in 25 diagnosis. Probes and primers are generally based on the nucleotide sequence of the genes. Hybridization probes can comprise the whole or a large part of the coding or complementary strand of the sequence. PCR primers are typically smaller and encompass 30 about between 10 and 50, preferably between 15 and 30, more preferably about 20 nucleotides.

The nucleotide sequences of some suitable PCR primers are given in the following table.

35 Table I

primer name	nucleotide sequence
F1RAP	5' GCA TCG TGG AAA GCA TGG CT 3'
F215RAP	5' GGG ACC CTT GAG AGA GCC TG 3'

F371RAP	5' CTT GAG GCA GCA GTT GAA AC 3'
F571RAP	5' TCC ACC CTT ATG CAG AAC GC 3'
F771RAP	5' AGT ACC TTC ATC CGT GTC AG 3'
F971RAP	5' CGC CTT CGC TCC AGA GTT GG 3'
5 F1171RAP	5' AGG GTG GAG GGT CCT GCA TA 3'
F1371RAP	5' GCA AGC CAG TAC TTG ACC GT 3'
F1621RAP	5' GTG GTC CTG CTG GGG GAT CA 3'
R234RAP	5' CAG GCT CTC TCA AGG GTC CC 3'
R420RAP	5' CTA CCT GCT CCA TCA GCT CG 3'
10 R570RAP	5' AGA GTT CTT TGA CTC GGT CC 3'
R770RAP	5' GAG CTC ATC TCG CAG CTG AT 3'
R970RAP	5' CTG TGG CTA GGC GGG GGT GG 3'
R1170RAP	5' CTG CCT ATT AGG CCA TGC TG 3'
R1370RAP	5' AGT CAG TCT CCC CCG CAC AC 3'
15 R1570RAP	5' TGG CAG GGA TGT ACA CAC TC 3'
R1837RAP	5' TTT CCA TCA TGA GCG TCT AT 3'

The hybridization probes can be labeled with a detectable label, such as a radioactive or biotin label.

20 Diagnosis of expression of the gene can be performed by means of a Northern blot. Total RNA or mRNA of a sample is separated on an agarose gel. The separation pattern is transferred to a nylon or nitrocellulose filter. An increase or decrease in the 25 expression level is subsequently detected by hybridization with the above described hybridization probe. Typically a reference sample is included for comparison.

In case the protein is the basic macromolecule 30 for diagnosis polyclonal or monoclonal antibodies are used for detection. The skilled person is very well capable of preparing such antibodies based on his common knowledge. Antibodies against the protein are part of the present invention.

35 Samples to be diagnosed can be a liver biopsy, plasma or serum. The latter can be used because the protein is secreted in the blood stream.

With the above described diagnostic methods an increase or decrease in the expression of the gene of the invention can be detected. The information that can thus be obtained is useful for establishing the efficacy of 5 therapeutic agents stimulating liver regeneration and for patients who underwent an (auxiliary) liver transplantation and for monitoring patients treated with a bioartificial liver.

The invention is further illustrated in the 10 following examples, which are in no way intended to be limiting to the invention. In the examples reference is made to the following figures:

Figure 1A is the nucleotide sequence of the 1282 bp cDNA.

15 Figure 1B is the nucleotide sequence of the 1834 bp cDNA.

Figure 2 shows the deduced amino acid sequence of the rap3 protein.

Figure 3 shows a polyacrylamide gel of liver 20 cDNA fragments before and after subtraction. 26 cDNA fragments were found to be enriched after subtraction. Some of these are indicated by arrows. Lane 1 shows liver cDNA fragments of 6 hours 70% partial hepatectomy before subtraction. Lane 2 shows cDNA fragments of 6 hours 70% 25 partial hepatectomy after subtraction.

Figure 4 shows the results of the Northern blot analysis of the temporal expression of RAP3 up to 30 hours after 70% partial hepatectomy. Panel A represents the Northern blot mRNA expression patterns at 3, 6, 12, 30 18, 24 and 30 hours after the 70% hepatectomy (hpx) and laparotomy (sham). Panel B represents the quantified hybridization signals indicated in PhosphorImager arbitrary units obtained at 6, 12, 18, 24 and 30 hours after the 70% hepatectomy and laparotomy.

35 The novel gene RAP3 is mostly upregulated 6 hours after partial hepatectomy after which it becomes downregulated.

Figure 5 shows a rat tissue Northern blot hybridized with a RAP3 cDNA probe. The RAP3 gene is specifically expressed in the liver.

Figure 6A shows the nucleotide sequence of the 5 1282 bp human RAP3 cDNA.

Figure 6B shows the nucleotide sequence of the 1867 bp human RAP3 cDNA (B).

Figure 7 shows the deduced amino acid sequence of the human rap3 protein.

10 Figure 8 shows the results of immunoblotting. About 6 ng of denatured rat RAP-3 protein was blotted on each strip. The strips were incubated with two antisera in various concentrations: 1-3: antiserum against purified native rat RAP3 protein (concentrations: 1:2000, 15 1:8000, 1:32000); 4-7: antiserum against purified denatured rat RAP3 protein (1:2000, 1:8000, 1:32000, 1:128000).

Figure 9 shows the results of an immunoblot after analysis with the program LumiAnalyst. 2.5  $\mu$ l rat 20 plasma was blotted per lane. The blot was incubated with antiserum against the denatured RAP3 protein. (hpx: plasma after 70% partial hepatectomy; sham: plasma after sham operation; positive control: about 10 ng purified denatured rat RAP3 protein).

25

**EXAMPLES**

**EXAMPLE 1**

**Isolation of RAP3 gene associated with liver regeneration**

30 **1. Introduction**

Recovery from Hepatic injuries caused by viruses, toxins, ischemia, surgery and auxiliary liver transplantation can be achieved by regeneration of the liver. The regeneration process has been studied 35 extensively in the rat after a 70% partial hepatectomy.

During the first four hours following partial hepatectomy there is a rapid, transient transcriptional response. After this induction during the transition from

the quiescent state of the liver ( $G_0$ ) to the growth phase ( $G_1$ ), a delayed early gene activation is initiated, which peaks during the transition of the  $G_1$  to the DNA synthesis phase (S phase).

5 This example demonstrates the isolation and identification of genes which are upregulated in the regenerating liver 6 hours after 70% partial hepatectomy.

## 2. Methods

### 10 2.1 Rat liver tissue preparation

Experiments were carried out in compliance with the guidelines on the care and use of laboratory animals of the University of Amsterdam. Regenerating liver was obtained from male Wistar rats (200-225 g). Rats were 15 anesthetized with ether and subjected to midventral laparotomy. Subsequently, the left lateral and the median liver lobes were removed (70% partial hepatectomy) (G.M. Higgins and R.M. Anderson, Arch. Pathol. 12, 186 (1931)). For sham-operated animals, the liver was exposed 20 by a midventral laparotomy.

The rats were allowed to recover from anesthesia. At 3, 6, 12, 18, 24, and 30 hours, respectively, after the 70% partial hepatectomy and sham surgery the animals were killed and the remaining liver 25 was immediately harvested.

## 2.2 RNA isolation

Total liver RNA was isolated from liver tissue using the Trizol reagent kit (Life Technologies). Liver poly A<sup>+</sup> RNA was isolated from total liver RNA using oligo-  
5 (dT)-cellulose (Boehringer Mannheim GmbH) affinity chromatography as described previously (Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular cloning: A laboratory Manual, Cold Spring Harbor, NY). To obtain highly pure poly A<sup>+</sup> RNA populations the oligo-(dT)-  
10 cellulose step was performed twice. The integrity of the poly A<sup>+</sup> RNA populations was determined on Northern blot by hybridization with glutathione-S transferase (data not shown).

## 15 2.3 PCR-select cDNA subtraction

The PCR-select cDNA subtraction kit (Clontech) was used to selectively amplify delayed early genes differentially expressed during liver regeneration. This method subtracts sequences common to both cDNA  
20 populations by suppressing undesirable PCR amplification, rather than by physically separating single stranded and double-stranded DNA. The 6 hours 70% partial hepatectomy liver poly A<sup>+</sup> population, containing the differentially expressed mRNA's, was compared with the 6 hours  
25 laparotomy liver mRNA population. Delayed-early genes start to appear 3 to 4 hours after the 70% partial hepatectomy. By using a laparotomy liver mRNA population rather than a normal liver mRNA population, the two populations were equalized for acute phase mRNA's, which  
30 are induced by the operation itself.

The PCR-select cDNA subtraction was performed according to the manufacturer's protocol with the following modifications. After two hybridizations, a nested PCR was used to selectively amplify the  
35 differentially expressed sequences. The second, nested PCR was performed in the presence of 0.5  $\mu$ M [ $\alpha$ -<sup>33</sup>P]dATP (1200 Ci/mmol, final volume 25  $\mu$ l). Subsequently, the amplified and differentially expressed cDNA fragments

were visualized on a denaturing 4% polyacrylamide DNA sequencing gel. An X-ray film (Biomax, Kodak) was exposed overnight to the unfixed, dried gel.

Figure 3 shows the results of the subtraction.  
5 Before subtraction (lane 1), the majority of the cDNA's were poorly identifiable, indicating the presence of many cDNA fragments of different molecular size. After subtraction (lane 2), 26 distinct cDNA fragments were observed as bands that were not apparent before  
10 subtraction.

#### 2.4 Isolation and identification of visualized cDNA fragments

The 26 cDNA fragments that became visible after  
15 PCR-select cDNA subtraction were excised from the dried polyacrylamide gel and heated to 100°C for 5 minutes. Subsequently, 25 µl of the aqueous cDNA extract was used to amplify the cDNA by PCR with the nested primers used in the PCR-select cDNA subtraction. The PCR product was  
20 ligated into pCR II (Invitrogen), transformed into INVαF' competent cells, and plated out on agar plates containing ampicillin and X-Gal. Of each cloned PCR product, 6 white colonies were analyzed by PCR with T7 and SP6 primers for the presence of an insert.

25 Subsequently, plasmids containing an insert were purified using QIAprep (Qiagen) and the sequences of the inserts were determined using a dye terminator cycle sequencing system (Perkin Elmer) and a 377 DNA sequencer (ABI PRISM).

30

#### 2.5 Northern blot analysis

To determine whether the expression of the genes found by the PCR-select subtractive hybridization is truly increased 6 hours after partial hepatectomy,  
35 Northern blot analysis was carried out using the purified cDNA fragments as probes. Poly A<sup>+</sup> RNA samples (0.8 µg) of the liver 6 hours after the hepatectomy and sham operation were electrophoresed on a 0.22 M formaldehyde-

1% agarose gel, and blotted onto a Hybond-N nylon membrane (Amersham) by capillary transfer overnight. For fixation of the poly A<sup>+</sup> RNA the blots were baked in an oven at 80°C for 2 hours.

5 The inserts of the sequenced clones were amplified by PCR using the nested primers of the PCR-select cDNA subtraction method. Qiaquick-spin columns (Qiagen) were used to purify the PCR products. The purified PCR products were radioactively labelled  
10 according to the hexamer-random primed method following the manufacturer's protocol (Promega), purified on Qiaquick-spin columns (Qiagen), and hybridized with the blots. Prehybridization (2 hours, 42°C) and hybridization (overnight, 42°C) was performed in 5 x SSPE, 50%  
15 formamide, 5 x Denhardt, 0.5% SDS, and 0.1 mg/ml sheared heat-denatured herring sperm DNA.

Following hybridization the blots were washed with 2 x SSC and 0.1% SDS for 15 min at room temperature and 42°C, respectively. Subsequently, the solution was  
20 replaced with 1 x SSC and 0.1% SDS and the blots were washed for 15 min at room temperature and at 42°C, respectively. The amount of hybridization was analyzed and quantified using a PhosphorImager (Molecular Dynamics).

25 The fold induction of the mRNA levels observed in the 70% partially hepatectomized animals over the sham operated animals after the specific hybridization was adjusted for variability in RNA loading.

30 The genes which were upregulated 1.5 times or more 6 hours after 70% hepatectomy together with their identity are given in Table II. Beside these twelve genes, three genes are indicated which expression could not be detected on Northern blot. The expression of the novel RAP3 gene was found to be upregulated 3.3 fold.

Table II

GENES UPREGULATED 6 HOURS AFTER A 70%			
	Identity of gene	Function	Fold
5	Fibronectin	Liver regeneration	1.8
	An intracisternal-A	Liver regeneration	1.8
	$\gamma$ -Actin	Liver regeneration	7
	Ribophorin I	Liver regeneration	5.5, 1.7 & 2.3
10	$\alpha_2$ -Macroglobulin	Hepatocyte proliferation <u>in vitro</u>	5.4
	Ribosomal Protein S5	Cell cycle	3.7 & 1.9
	Ribosomal Protein	Cell cycle	2
	L13		
15	Amyloid A Protein	Growth factor	10.4
	Entactin		N.D.*
	TCP-1-Containing		1.5
	Chaperonin related gene		
20	31 kDa Putative		N.D.*
	Serine/ Threonine protein kinase		
	Novel RAP1**	Unknown	1.5
	Novel RAP2**	Unknown	1.6
25	Novel RAP3**	Unknown	3.3
	Novel RAP4**	Unknown	N.D.*

\* N.D. = not detectable on Northern blot

\*\* RAP1-3: Regeneration Associated Protein1-3)

### 30 EXAMPLE 2

#### Isolation and characterization of the full length RAP3

#### CDNA

#### Library screening and sequence analysis

A rat liver cDNA library was prepared from poly  
35 A<sup>+</sup> RNA isolated from the rat liver 6 hours after 70%  
hepatectomy. To obtain full length cDNA, the Great  
Lengths cDNA Synthesis Kit (Clontech) was used following  
the manufacturer's protocol. The adaptor ligated full

length cDNA inserts were cloned into the mammalian expression vector pCI at the EcoRI restriction site.

After transformation into DH10B electrocompetent cells (Gibco), the cDNA library was 5 plated at a density of about 3,000 plaques per 150-mm-diameter petri dish. Colonies were lifted onto a Hybond-N nylon membrane (Amersham). The lift was hybridized with the <sup>32</sup>P-labeled RAP3 PCR fragment prepared according to the hexamer-random primed method following the 10 manufacturer's protocol (Promega).

Following hybridization, the lift was washed and analyzed using a PhosphorImager (Molecular Dynamics). From the nine positive clones, the plasmid DNA was purified and the sequences of the inserts were determined 15 using a Big dye terminator cycle sequencing system (Perkin Elmer) and an ABI PRISM 377 DNA sequencer (Perkin Elmer). The RAP3 cDNA was obtained by comparing the nine sequences with the sequence of the RAP3 PCR fragment. Two possible clones were detected and the start and end of 20 the cDNA were confirmed by 5'- and 3'-RACE reactions carried out following the protocol of the Marathon cDNA Amplification kit (Clontech).

Based on the nucleotide sequence of the clones, PCR reactions were carried out with cDNA prepared from 25 poly A<sup>+</sup> RNA of the rat liver 6 hours after 70% hepatectomy. The PCR products comprised the whole RAP3 cDNA, of which the nucleotide sequence was determined by bidirectionally sequencing the PCR products using 20 bp primers based on the already known nucleotide sequence 30 data of the RAP3 cDNA.

Two RAP3 cDNA molecules were detected of 1282 and 1834 bp respectively. The latter showed the same nucleotide sequence as the first, but contained an additional 552 bp nucleotide part at the 3' side.

35 The nucleotide sequence of the 1282 bp RAP3 cDNA is as shown in Figure 1A.

The nucleotide sequence of the 1834 bp RAP3 cDNA is shown in Figure 1B.

Using GCG DNA software the nucleotide sequences were translated into the amino acid sequence. By analyzing the six reading frames, the largest possible protein was chosen as the RAP3 protein. Its amino acid sequence, starting with a methionine residue and ending at a stop codon, was the most likely one to form a protein in comparison with the other smaller possible proteins. Both RAP3 cDNA molecules encode the same RAP3 protein.

10 The amino acid sequence of RAP3 protein as deduced from the nucleotide sequence is shown in Figure 2.

15 **EXAMPLE 3**

Temporal expression between 3 and 30 hours after 70% partial hepatectomy

To define the temporal expression of the RAP3 gene, mRNA levels at 3, 6, 12, 18, 24, and 30 hours after 20 the 70% partial hepatectomy and laparotomy were analyzed by the Northern blot procedure as described in example 1. Total RNA samples (20 µg) of the rat liver isolated at the various time points were electrophoresed. The Northern blot was hybridized with a radioactively labeled 25 probe comprising basepairs 370 to 1834 of the large RAP3 cDNA. The result of the Northern blot and the quantified expression pattern are given in Figure 4. The expression pattern is presented as the hybridization signal in PhosphorImager arbitrary units obtained at 3, 6, 12, 18, 30 24, and 30 hours after the 70% partial hepatectomy and laparotomy.

Both RAP3 mRNA sizes are mostly upregulated 6 hours after partial hepatectomy after which they become downregulated.

35 The same procedure was carried out with probes of the other upregulated genes obtained by the PCR-select subtraction. Two distinct gene expression patterns during the 30 hour period after partial hepatectomy were found.

The first pattern has two peaks coincident with the G<sub>1</sub> phases of two consecutive hepatic cycles. The second one shows a narrow peak at six hours after which the gene is downregulated, just like the expression pattern of the 5 novel RAP3 gene.

Determination of tissue specific expression

A Northern blot was prepared to determine expression of RAP3 mRNA in different tissues. The various 10 tissues (skeletal muscle, spleen, liver, kidney, heart, lung and brain) were isolated from a female Wistar rat (175 g). The experiment was carried out in compliance with the guidelines on the care and use of laboratory animals of the University of Amsterdam. Total liver RNA 15 was isolated from the tissues using the Trizol reagent kit (Life Technologies). A Northern blot was prepared from 20 µg total RNA samples and Northern blot analysis was carried out as described in example 1. A radioactively labeled probe comprising basepairs 370 to 20 1834 of the large RAP3 cDNA was used for the hybridization. The resulting Northern blot is given in Figure 5.

The RAP3 mRNA appeared to be clearly expressed in the liver and not at any detectable level in the other 25 examined tissues. Because of this liver specificity and the 3.3 fold upregulation six hours after hepatectomy, the novel gene RAP3 was considered to be important in the process of liver regeneration.

30

**EXAMPLE 4**

Isolation and characterization of the full length human RAP3 cDNA

The Superscript Human liver cDNA library was 35 purchased from Life Technologies. It contained liver cDNA from a 9-year old caucasian female directionally cloned into the pCMV<sup>1</sup>SPORT vector, and transformed into DH12S cells.

The cDNA library was plated at a density of about 25,000 plaques per 150-mm-diameter petri dish. Colonies were lifted onto a Hybond-N nylon membrane (Amersham). The lift was hybridized with a <sup>32</sup>P-labeled RAP3 cDNA fragment, 5 consisting of the overlapping nucleotide sequence of the two rat RAP3 cDNA molecules. The labeling was carried out according to the hexamer random primed method following the manufacturer's protocol (Promega). Following hybridization, the membrane was washed with low 10 stringency and analyzed using a PhosporImager (Molecular Dynamics).

Eight positive colonies and its near surroundings were cut from the agar plate and each transferred into 1 ml LB medium. 20  $\mu$ l was plated on new 15 agar plates and the hybridization protocol was repeated. Duplo clones of the eight positive colonies were isolated from the plate. The plasmid DNA was purified and the sequences of the inserts were determined using a Big Dye terminator cycle sequencing system (Perkin Elmer) and an 20 ABI PRISM 377 DNA sequencer (Perkin Elmer). For sequencing initially the T7 and SP6 promotor primers were used and later on primers identical to twenty nucleotides of the sequenced part of the inserts.

By bidirectionally sequencing two human RAP3 cDNA 25 nucleotide sequences were detected, both being presented by four of the eight examined clones. The two RAP3 cDNA molecules were 1322 and 1867 bp respectively. The latter showed the same nucleotide sequence as the first, but contained an additional 545 bp nucleotide part at the 3' 30 side. The 1867 human RAP3 cDNA shows a 76% identity with the 1834 bp rat RAP3 cDNA.

The nucleotide sequence of the 1282 bp human RAP3 cDNA is shown in Figure 6A. The nucleotide sequence of the 1867 bp human RAP3 cDNA is shown in Figure 6B.

35 Using GCG DNA software the nucleotide sequences were translated into the amino acid sequence. By analyzing the six reading frames, the largest possible protein was chosen as the human RAP3 protein. Its amino

acid sequence, starting with a methionine residue and ending at a stop codon, was the most likely one to form a protein in comparison with the other smaller possible proteins. Both human rRAP3 cDNA molecules encode the same 5 rap3 protein. The human RAP3 protein shows a 73% identity with the rat RAP3 protein, indicating that they indeed are the same protein but only expressed in different species.

The 363 residues counting amino acid sequence 10 of the human RAP3 protein as deduced from the nucleotide sequence is shown in Figure 7.

#### EXAMPLE 5

15 Production of polyclonal antibodies against both rat and human RAP3

##### Expression of RAP3 protein

Both the rat and human cDNA sequences encoding 20 the RAP3 protein without its presequence (the first 20 amino acid residues of the protein) were cloned into the pET-15b expression vector (Novagen) at the NdeI restriction site. The inserts were flanked by NdeI restriction sites, which had been introduced by PCR. By 25 cloning at the NdeI site, a His-taq is expressed in front of the RAP3 protein, which is necessary for purification of the protein.

The vectors containing the RAP3 insert were transformed into the bacterial strain BL21(DE3) 30 (Novagen). One colony was inoculated in 200 ml ampicillin containing LB medium. The medium was incubated at 37 °C. After reaching an OD<sub>600</sub> of 0.6/0.7 the medium was incubated for another four hours after which the cells were isolated.

35 The rat RAP3 protein was purified under non-denaturing conditions from the soluble fraction of the cells and under denaturing conditions from the insoluble fraction (inclusion bodies) of the cells. The human RAP3

protein was only purified under denaturing conditions from the insoluble fraction. The purification was carried out using His-Bind resin and columns (Novagen) following the manufacturer's protocol.

5 For identification the purified proteins were sequenced. Therefore the His-tag was removed from the proteins by cleavage with biotinylated thrombin (Novagen) following the manufacturer's protocol. The proteins were run on a 10% SDS-PAGE gel in Laemmli running buffer. The 10 gel was blotted onto a PVDF membrane in CAPS buffer. The blot was shortly stained with Fast Blue (Pharmacia). The proteins were sequenced from blot by the Protein Research Facility Amsterdam of the E.C. Slater Institute in Amsterdam. The first thirteen amino acid residues of the 15 expressed proteins after the His-tag were identified to be the same as the amino acid residues 21-33 of the rat and human RAP3 proteins as depicted in Figure 2 and 7 respectively. This indicates that the expected RAP3 proteins were expressed and can be used as antigens to 20 raise polyclonal antibodies against the RAP3 proteins.

#### Immunisation

Rabbits were immunised with the various RAP3 proteins in order to obtain polyclonal antibodies against 25 the rat and human RAP3 protein. About 200 µg purified RAP3 protein (including the His-tag) was suspended in Freund Complete Adjuvant and injected intracutaneously into 15 weeks old New Zealand White rabbits. After 2 months a booster of about 200 µg protein in Freund 30 Incomplete Adjuvant was given intramuscular. One month later a booster of about 200 µg protein in PBS was given intramuscular and one to two weeks hereafter serum was collected from the rabbits.

The serum was tested in various concentrations for its 35 ability to recognize the purified RAP3 protein. The sera against rat RAP3 were tested on the purified denatured rat RAP3 protein by immunoblotting. About 45 ng protein was run on a 10% SDS-PAGE gel in Laemmli running buffer.

The gel was blotted onto a PVDF membrane in Laemmli running buffer containing 20% methanol. The blot was cut into seven strips each loaded with approximately 6 ng rat RAP3 protein. The strips were blocked using Protifar 5 (Nutricia), washed and incubated with antibodies (1 h, 20 °C) according standard methods. The strips were incubated first with the sera against the rat RAP3 protein in various concentrations ranging from 1:2,000 to 1:128,000. Secondly the strips were incubated with alkaline 10 phosphatase labeled goat anti-rabbit immunoglobulins (DAKO). The strips were now incubated with precipitating BM purple AP substrate (Boehringer Mannheim) following the manufacturer's protocol. Reaction of alkaline phosphatase with the substrate leaves a dark purple band. 15 The results are given in Figure 8. As shown a clear band at the right protein size appears even at the lowest concentrations. Serum from the rabbits before immunisations did not react with the RAP3 protein (data not shown). So polyclonal antibodies were obtained 20 against the rat RAP3 protein. In the same way polyclonal antibodies against human RAP3 will be obtained.

#### EXAMPLE 6

##### 25 Detection of RAP3 protein in rat plasma

###### Rat plasma isolation

Experiments were carried out in compliance with the guidelines on the care and use of laboratory animals 30 of the University of Amsterdam. Regenerating liver was obtained from male Wistar rats (200-225 g). Rats were anesthetized with Hypnorm and subjected to midventral laparotomy. Subsequently, the left lateral and the median liver lobes were removed (70% partial hepatectomy) {G.M. 35 Higgins and R.M. Anderson, Arch. Pathol. 12, 186 (1931)}. For sham-operated animals, the liver was exposed by a midventral laparotomy. The rats were allowed to recover from anesthesia. At 3, 6, 12 hours, respectively, after

the 70% partial hepatectomy and sham surgery the blood of the animals was heparinized and collected. The plasma of the rats was obtained by 5 minutes centrifugation at 1650g.

5

#### Immunoblotting

A Western blot was prepared as described before containing 2.5  $\mu$ l plasma samples. Plasma samples of 3, 6 and 12 hours after both a sham operation and a 70% partial hepatectomy were examined. The blot was blocked (using Protifar), washed and incubated with antibodies (1 h, 20 °C) according standard methods. The blot was incubated first with serum against the denatured rat RAP3 protein in a 1:6,000 dilution. Secondly the strips were incubated with horse radish peroxidase labeled goat anti-rabbit immunoglobulins (DAKO). The blot was now incubated with Lumi-light<sup>plus</sup> Western blotting Substrate (Boehringer Mannheim) following the manufacturer's protocol. Analysis with the program LumiAnalyst gave the result as shown in Figure 9. RAP3 was found to be present in the plasma, so the liver excretes RAP3 into the blood. The appearing RAP3 has a somewhat smaller molecular weight than the positive control, because the positive control contains a His-tag in front of the protein. Especially the plasma samples of 6 and 12 hours after the hepatectomy showed an increase in RAP3 protein contents compared to the sham operation. So the concentration of the RAP3 protein is elevated in rat blood after 6 to 12 hours after a 70% hepatectomy.

30

#### **EXAMPLE 7**

##### Detection of changes of the amount of the RAP3 protein in the blood circulation

35 In order to detect changes in the amount of the RAP3 protein in the blood circulation a specific enzyme-linked immunosorbent assay (ELISA) is developed. Specific polyclonal and/or monoclonal antibodies are raised

against the whole protein or a part of the protein. The protein, human or rat, is expressed in a prokaryotic or eukaryotic expression system or part of the protein is synthesized chemically. Monoclonal and polyclonal 5 antibodies, raised in rabbits, are isolated by common techniques as described previously (Coligan, J.E., Kruisbeek, A.M., Margulies, D.M., Shevach, E.M., and Strober, W. (1994) Current Protocols in Immunology, John Wiley & Sons, Inc. Chichester, New York).

**CLAIMS**

1. Gene involved in regeneration processes of the liver and comprising a nucleotide sequence which is at least 70% homologous to the sequence shown in Fig. 1 or at least 70% homologous to the sequence shown in Fig. 6, or the complementary strand thereof.
2. Gene as claimed in claim 1, characterized in that its cDNA has a nucleotide sequence which is at least 70% homologous to the nucleotide sequence as depicted in Fig. 1 or at least 70% homologous to the nucleotide sequence as depicted in Fig. 6, or the complementary strand thereof.
3. Gene as claimed in claims 1 or 2 for use in the design of PCR probes for detecting nucleotide sequences in a source material, which nucleotide sequences represent genes corresponding with the gene sequence shown in Fig. 1 or with the gene sequence shown in Fig. 6.
4. Gene as claimed in claims 1 or 2 for use as a marker of liver proliferation.
5. Protein encoded by a gene as defined in claims 1 and 2 and comprising an amino acid sequence which is at least 70% homologous to the amino acid sequence given in Fig. 2 or at least 70% homologous to the amino acid sequence given in Fig. 7.
6. Protein as claimed in claim 5 having the amino acid sequence as depicted in Fig. 2 or Fig. 7.
7. Protein as claimed in claims 5 and 6 for use in diagnosis of liver regeneration and/or liver cell proliferation.
8. Antibodies directed against a protein as claimed in claims 5 and 6.
9. Antibodies as claimed in claim 7 for use in a method for detecting the occurrence of liver cell proliferation in a subject.
10. Antibodies as claimed in claim 8 or 9 which antibodies are monoclonal antibodies.

11. Antibodies as claimed in claim 8 or 9 which antibodies are polyclonal antibodies.

12. PCR primer, comprising at least part of the gene as claimed in claim 1.

13. PCR primer, comprising at least part of the nucleotide sequence as shown in Fig. 1 or at least part of the nucleotide sequence as shown in Fig. 6, or its complementary strand.

14. PCR primer as claimed in claims 12 and 13, wherein the "at least part of the nucleotide sequence" encompasses 10 to 50, preferably 15 to 30, more preferably about 20 nucleotides.

15. PCR primer as claimed in claims 12 to 14 having the nucleotide sequence as depicted in Table I or the complementary strand thereof.

16. PCR primer as claimed in claims 12 to 15 for use as a probe in a method for detecting the occurrence of liver proliferation in a subject.

17. PCR primer as claimed in claims 12 to 15 for use in the detection of gene homologous to the gene as claimed in claims 1 to 3.

18. Single stranded nucleotide sequence being at least in part complementary to the messenger RNA transcribed from a gene as claimed in claims 1 to 3.

19. Single stranded nucleotide sequence as claimed in claim 18 which is antisense RNA.

20. Single stranded nucleotide sequence being at least in part complementary to the DNA or the cDNA from a gene as claimed in claims 1 to 3.

21. Single stranded nucleotide sequence as claimed in claims 18-20, further provided with a detectable label.

22. Nucleotide sequence as claimed in claims 18 to 21 for use as a probe in a method for detecting the occurrence of liver proliferation in a subject.

23. Nucleotide sequence as claimed in claim 22, characterized in that the method in which the nucleotide sequence is used as a probe comprises the steps of:

- a) obtaining a sample of a tissue or body fluid; and
  - b) detecting the amount of messenger RNA transcribed from a gene as claimed in claims 1 to 3 in that sample in comparison to a reference sample by means of the probe.
24. Nucleotide sequence as claimed in claim 23, wherein the sample is a liver biopsy, plasma or serum.
25. Nucleotide sequence as claimed in claim 18, 20 or 21 for use as a probe for screening a liver cDNA or genomic library.
26. Nucleotide vector comprising the nucleotide sequence as claimed in claims 18 to 25.
27. Expression vector comprising the nucleotide sequence as claimed in claims 18 to 25 in operative association with a regulatory element that controls expression of the nucleotide sequence in a host cell.
28. Host cell comprising the nucleotide sequence as claimed in claim 18-25.
29. Host cell comprising the nucleotide sequence as claimed in claim 18-25 in operative association with a regulatory element that controls expression of the nucleotide sequence in that host cell.
30. Transgenic animal in which the gene as claimed in claim 1 or 2 is an expresses transgene comprised in the genome of the animal.
31. Transgenic animal in which expression of the gene as claimed in claim 1 or 2 is prevented or repressed.
32. Method for treating liver disorder, comprising administering a compound that modulates the expression of the gene as claimed in claim 1 or 2, or the activity of the gene product of that gene to a patient in need of such treatment.
33. Method as claimed in claim 32 wherein the compound is an antisense or ribozyme molecule that blocks translation of the target gene.

34. Method as claimed in claim 32 wherein the compound is complementary to the 5' region of the target gene and blocks transcription via triple helix formation.

35. Method as claimed in claim 32 wherein the compound is an antibody that neutralizes the activity of the target gene product.

36. Method as claimed in claim 32 wherein the compound enhances the expression of the target gene, or the synthesis or activity of the gene product.

37. Method for treating liver disorder comprising administering nucleic acid encoding the gene as claimed in claim 1 or 2 to a patient in need of such treatment.

38. Method for treating liver disorder comprising administering an effective amount of the gene product of the gene as claimed in claim 1 or 2 to a patient in need of such treatment.

39. Method for enhancing the growth or regeneration of liver tissue comprising treating the liver tissue with an effective amount of the gene product of the gene as claimed in claim 1 or 2.

40. Method as claimed in claim 39 wherein the liver tissue is extracorporeal.

41. Method as claimed in claim 39 wherein the liver tissue is intracorporeal.

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1	GCATCGTGG	AAGCATGGCT	GCCGTCA	CCTGGGC	CGCCCTC
51	TCAGTGT'TTC	CAACTGTACA	GGCGAGGAAG	AGCTTCTGG	AGTACTTC
101	CCAGAACAGC	CAGGGCAAAG	GCATGATGGG	CCAGCAGCAG	AAGCTGGC
151	AGGAGAGCCT	GAAAGGTAGC	TTGGAGCAAG	ACCTCTACAA	TATGAACAA
201	TTCCTAGAAA	AGCTGGGACC	C'TTGAGAGAG	CCTGGGAAGG	AGCCTCCTCG
251	GCTGGCACAG	GATCCAGAAG	GCATTGGAA	GCAGTTGCAG	CAAGAGCTGG
301	AGGAAGTGAG	CACACGCC	GAGGCC	TGGCTGC	AAA GCACCCAG
351	GTGGGCTGG	ACCTGGAGGG	C'TTGAGGCAG	CAGTTGAAAC	CCTACACGGT
401	CGAGCTGATG	GAGCAGGTAG	GCCTGAGCGT	GCAGGATCTG	CAAGAACAGC
451	TGGCGATGGT	GGGAAAGGC	ACCAAGGCC	AGCTCCTGG	GGCGGTGGAT
501	GAGGGCATGA	GCCTGCTGCA	GGATATGCAA	AGTCGAGTGC	TGCACCATA
551	GGACCCAGTC	AAAGAAACTCT	TCCACCC	TGGCAACGC	TGGTGA
601	GAATTGGCA	CCATGTGCAG	GAGCTGCACC	GGAGTGTGC	TCCTCACGCA
651	GTTGCCAGCC	CCGGGAGACT	CAGTCGCTGC	GTGCA	GACCC TGTCCCACAA
701	ACTCACACGT	AAGGGGAAGG	ACTTGCACAC	CAGGCATCAA	CGCAACCTGG
751	ATCAGCTGCG	AGATGAGCTC	AGTACCTTCA	TCCGGTGTCA	CACAGACGGG
801	GCAGACAAACA	GAGACTCCCT	GGACCCCTCAA	GCTCTCTCG	ACGAGGGTCCG

FIG. 1A-1

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851 CCAGAGACTC CAGGCTTTTC GACATGACAC CTACCTGCAG ATCGCTGCAT  
901 TCACTCAGGC CATTGACCAAG GAGACCGAGG AAATCCAGCA CCAGCTGGCA  
951 CCACCCCCGC CTAGCCACAG CGCCTTCGCT CCAGAGTTGG GACACTCAGA  
1001 CAGTAATAAG GCCCTGAGCA GACTGCAGAG CCGGCTGGAC GACCTCTGGG  
1051 AAGATATTGC CTATGGCTT CATGACCAGG GCCATAGTCA GAATAACCCCT  
1101 GAGGGTCACT CAGGTTAACT CTGCAGGCTCG TTGTCTGGAC CCTGAGGCCTT  
1151 CAGCATGGCC TAATAGGCAG AGGGTGGAGG GTCCCTGCATA CTATTGGCGA  
1201 GGCCACCAA GGTGCTGCTG CCCAACCTG TCTGGCCTCC TCAACTCCCC  
1251 CACTCAGGTG CATTACACTC AGTAGGTTG GC

FIG. 1A-2

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1    GCATCGTGG    AAGCATGGCT    GCCGTCA    CCTGGGCACT    CGCCCTCCTC  
51    TCAGTGTGG    CAACTGTACA    GGGGAGGAAG    AGCTTCTGGG    AGTACTTCGG  
101    CCAGAACAGC    CAGGGAAAG    GCATGATGGG    CCAGGAGCAG    AAGCTGGCAC  
151    AGGAGAGCCT    GAAAGGTAGC    TTGGAGGCAAG    ACCTCTACAA    TATGAAACAAT  
201    TTCCCTAGAAA    AGCTGGGACC    CTTGAGGAGAG    CCTGGGAAGG    AGCCTCCTCG  
251    GCTGGCACAG    GATCCAGAAG    GCATTGGAA    GCAGTTGCAG    CAAGAGCTGG  
301    AGGAAGTGAG    CACACGGCTG    GAGGCCCTACA    TGGCTGCAA    GCACCCAGCAG  
351    GTCGGCTGG    ACCTGGGGG    CTTGAGGCAG    CAGTTGAAAC    CCTACACGGT  
401    CGAGCTGATG    GAGCAGGTAG    GCCTGAGCGT    GCAGGGATCTG    CAAGAACAGC  
451    TGCGCATGGT    GGGAAAGGC    ACCAAGGCC    AGCTCCTGGG    GGGCGTGGAT  
501    GAGGCCGATGA    GCCTGCTGCA    GGATATGCAA    AGTCGAGTGC    TGCACCATAC  
551    GGACCCGAGTC    AAAAGAACTCT    TCCACCCCTTA    TGCAGAACGC    TTGGGTGACTG  
601    GAATTGGCA    CCATGTGCAG    GAGCTGCACC    GGAGTGTGTC    TCCTCACCGCA  
651    GTTGGCCAGCC    CCGGGAGACT    CAGTCGGCTGC    GTGCAGACCC    TGTCCCCACAA  
701    ACTCACACGT    AAGGCGAAGG    ACTTGCACAC    CAGCATCCAA    CGCAACCTGG  
751    ATCAGCTGCG    AGATGAGCTC    AGTACCTTCA    TCCGTGTCA    CACAGACGGG  
801    GCAGACAACA    GAGACTCCCT    GGACCCCTCAA    GCTCTCTCG    ACGAGGGTCCG

FIG. 1B-1

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851	CCAGGAGACTC	CAGGCTTTTC	GACATGACAC	CTACCTGCAG	ATCGCTGCAT
901	TCACTCAGGC	CATTGACCA	GAGACCGAGG	AAATCCAGCA	CCAGCTGGCA
951	CCACCCCCGC	CTAGCCACAG	CGCCCTTCGCT	CCAGAGTTGG	GACACTCAGA
1001	CAGTAATAAG	GCCCTGAGCA	GACTGCAGAG	CCGGCTGGAC	GACCTCTGGG
1051	AAGATATTGC	CTATGGCCTT	CATGACCA	GAATACCCCT	
1101	GAGGGTCACT	CAGGTTAACT	CTGCAAGCTCG	TTGTCTGGAC	CCTGAGGCCTT
1151	CAGCCATGGCC	TAATAGGCAG	AGGGTGGAGG	GTCCCTGCATA	CTATTGGCGA
1201	GGCCACCAAA	GGTGCTGCTG	CCCCAACCTG	TCTGGCCTCC	TCAACTCCCC
1251	CACTCAGGTG	CATTACACTC	AGTAGGTTTG	GCAAACACAG	CTTCCGGTGC
1301	TCATTGGGA	TCCTAAGGAG	CAAGAGTGGG	GTGAAGGGAG	TGGGGAGATG
1351	GTGTGCGGGG	GAGACTGACT	GCAAGCCAGT	ACTTGACCGT	TGCTAGAAC
1401	CTGTTGTCACT	ACAAACCTGGA	GCCCCGGCTCC	TATTACTTCA	TGCCTGATGG
1451	TCGCTGTTAT	AGTCGGTCTA	CAGAGGGAA	CTCCTGTCTC	CCCAGGGTTG
1501	TCATGACAGC	CTTTGTTGGA	AGAGAGGAGG	AGAACATGAC	ACGTATGATG
1551	GAGTGTGTAC	ATCCCTGCCA	GTGGGTCCCTGC	TGGGGAAATC	AGTGATGGGA
1601	TAATATGTTG	CATCCCTTGCA	GTGGGTCCCTGC	TGGGGATCA	GTGATGGGAT
1651	GGGGCAGAGC	CCCTTATTTCC	TTAGAGAACT	CTAACCCAAA	TAAGGAACTG

FIG. 1B-2

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1701 AGCCCTCTGC AGTGAGGGCT TCTGAAAACC CTGTACATAG CAAACTGTGT  
 1751 GCCCTCTTCA TCATGCAGTC CCCACCTCCT GATTCTCGGG ATGGAACTGA  
 1801 CTTTGTTTG GAATGAAATA GACGGCTCATG ATGG

FIG. 1B-3

1 MAAVITWALLA LLSVFATVQA RKSFWEYFGQ NSQGKGMMGQ QQKLAQESLK  
 51 GSLEQDLYNM NNFLEKIGPL REPGEKEPPRL AQDPEGIRKQ LQQELEEVST  
 101 RLEPYMAAKH QQVGNLLEGQ RQQLKPYTYE LMEQVGLSVO DLQEQLRMVG  
 151 KGTRKAQLLGG VDEAMSLLQD MQSRLVLLHHTD RYKELFHFPYA ERLVITGIGHI  
 201 VQELHRSVAP HAVASPARLS RCVQTLSSHKL TRKAKDLHTS IQRNLDDQLRD  
 251 ELSTFIRVST DGADNRDSDL PQALSDEVRQ RLQAFRHDTY LQIAAFTQAI  
 301 DQETEEIQHQ LAPPPPSSHSA FAPELGHSDS NKALSKLQSK LBBLWEBDAY  
 351 GLHDQGHSON NPEGHSG\*

FIG. 2

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EPO - DG 1

27. 09. 1999

(67)

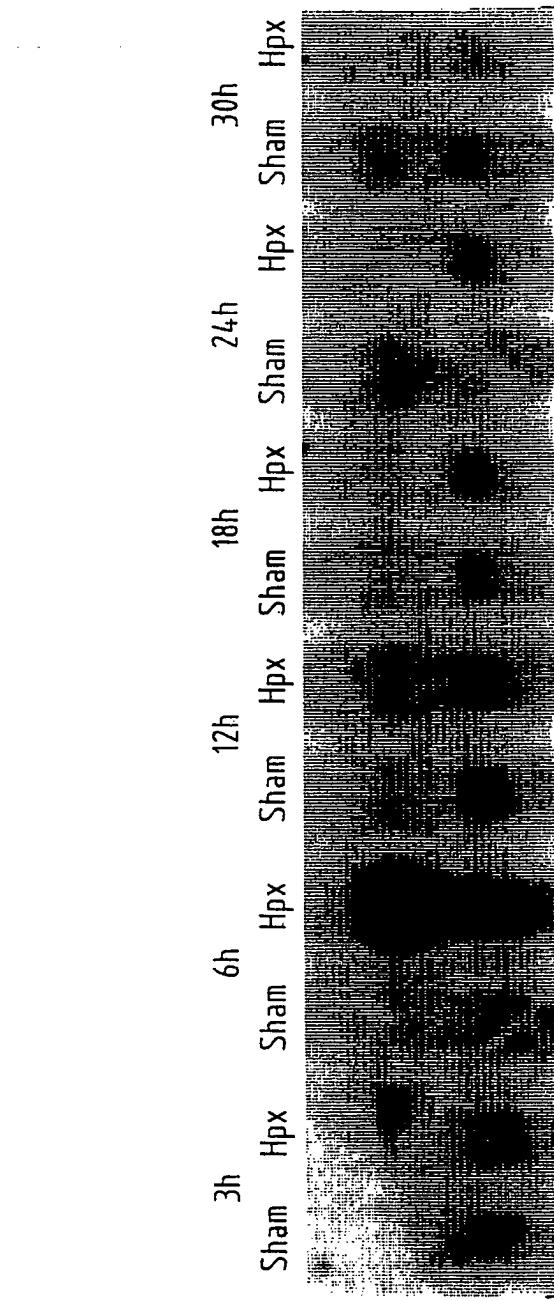


FIG. 4A

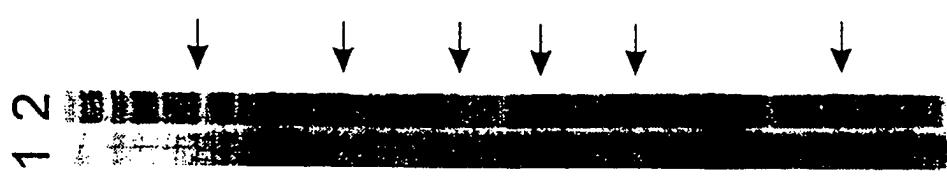


FIG. 3

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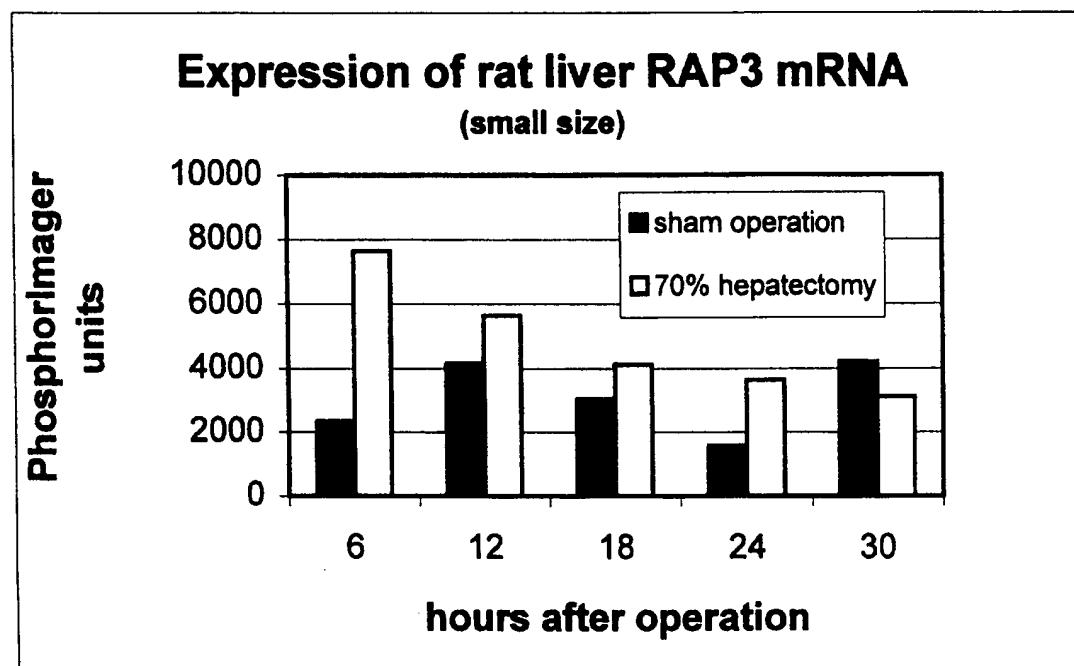
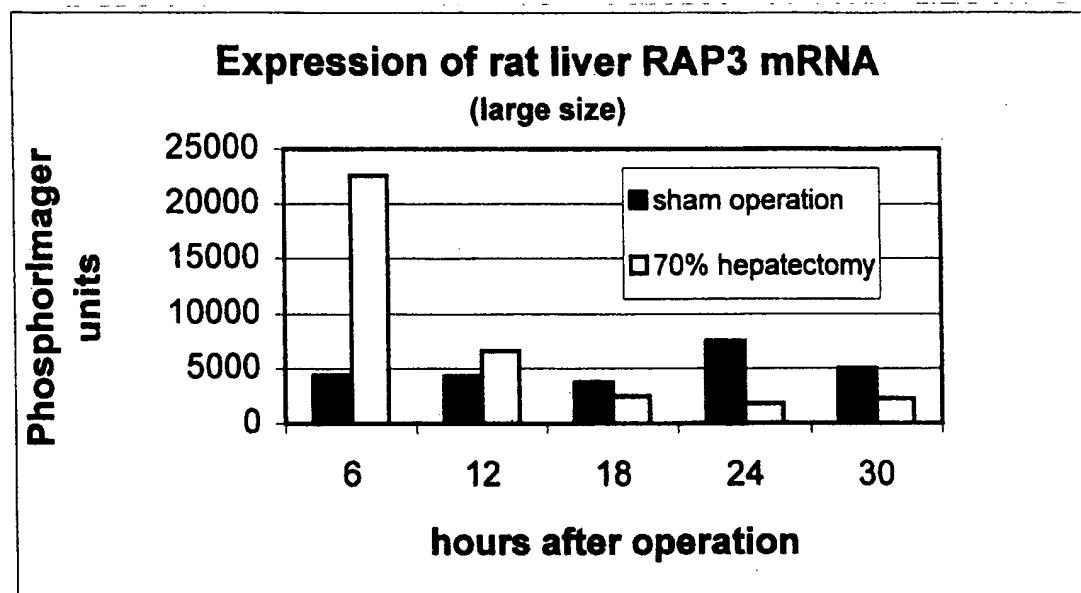


FIG. 4B

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Skeletal Spleen Liver Kidney Heart Lung Brain  
muscle



FIG. 5

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1    GAGCAGATAA    TGGCAAGGCAT    GGCTGCCGTG    CTCACCTGGG    CTCTGGCTCT  
51    TCTTTCAGCG    TTTTCGGCCA    CCCAGGCACG    GAAAGGCTTC    TGGGACTACT  
101    TCAGCCAGAC    CAGCGGGAC    AAAGGCAGGG    TGAGGCAGAT    CCATCAGGAG  
151    AAGATGGCTC    GCGGAGCCCGC    GACCCCTGAAA    GACAGCCTTG    AGCAAGACCT  
201    CAAACAATATG    ACAAAGTTCC    TGGAAAAGCT    GAGGCCTCTG    AGTGGGAGCG  
251    AGGCTCCTCG    GCTCCACAG    GACCCCGGTGG    GCATATGGCGCG    GCAGGCTGGCAG  
301    GAGGAGTTGG    AGGAGGTGAA    GGCTCGGCCTC    CAGGCCCTACA    TGGCAGAGGC  
351    GCACGAGCTG    GTGGGCTGGA    ATTGGAGGG    CTTGGAGGG    CAACTGAAGC  
401    CCTACACGAT    GGATCTGATG    GAGGCAAGGTGG    CCCTGCCGT    GCAGGGAGCTG  
451    CAGGAGCAGT    TGGCGCTGGT    GGGGGAAGAC    ACCAAGGCC    AGTTGCTGGG  
501    GGGCGTGGAC    GAGGCTTGGG    CTTTGCTGCA    GGGACTGCAG    AGCCCGCGTGG

FIG. 6A-1

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551 TGCACCCAC CGGCCGCTTC AAAGAGCTCT TCCACCCATA CGCCCGAGAGC  
601 CTGGTGAGCG GCATCGGGCG CCACGTGCAG GAGGCTGCACC GCAGTGTGGC  
651 TCCGCACGCC CCCGCCAGGCC CCGGGCGCCT CAGTCGCTGC GTGCAGGGTGC  
701 TCTCCGGAA GCTCACGGCTC AAGGCCAAGG CCCTGCACGC ACGCATCCAG  
751 CAGAACCTGG ACCAGCTGCG CGAACAGGCTC AGCAGAGCCT TTGCAGGCAC  
801 TGGGACTGAG GAAGGGGCCG GCCCGGACCC CCAGATGCTC TCCGAGGGAGG  
851 TGCCGCCAGCG ACTTCAGGGCT TTCCGGCCAGG ACACCTACCT GCAGATAAGCT  
901 GCCTTCACTC GCGCCATCGA CCAGGGAGACT GAGGAGGTCC AGCAGGCAGCT  
951 GGCGCCACCT CCACCAAGGCC ACAGTGCCCT CGCCCCAGAG TTTCAACAAA  
1001 CAGACAGTGG CAAGGTTCTG AGCAAGCTGC AGGCCCCGTCT GGATGACCTG  
1051 TGGGAAGACA TCACTCACAG CCTTCATGAC CAGGGCCACA GCCATCTGGC

FIG. 6 A - 2

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1101 GGACCCCTGA GGATCTACCT GCCCAGGCC ATTCCCCAGCT CCTTGTCTGG  
1151 GGAGCCTTGG CTCTGAGCCT CTAGCATGGT TCAGTCCTTG AAAGTGGCCCT  
1201 GTTGGGTGGA GGGTGAAGG TCCITGTGCAG GACAGGGAGG CCACCAAAGG  
1251 GGCTGCTGTC TCCTGCATAT CCAGCCTCCT GCGACTCCCC AATCTGGATG  
1301 CATTACATTC ACCAGGCTTT GC

FIG. 6A-3

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1 GAGCAGATAA TGGCAAGCAT GGCTGCCGTG CTCACCTGGG CTCTGGCTCT  
51 TCTTTCAGCG TTTTCGGCCA CCCAGGCACG GAAAGGCCTTC TGGGACTACT  
101 TCAGCCAGAC CAGCGGGAC AAAGGCAGGG TGAGGCAGAT CCATCAGCAG  
151 AAGATGGCTC GCGAGCCCCGC GACCCCTGAAA GACAGCCCTTG AGCAAGACCT  
201 CAACAATATG ACAAAGTTCC TGGAAAAGCT GAGGCCTCTG AGTGGGAGCG  
251 AGGCCTCCTCG GCTCCCACAG GACCCGGTGG GCATGCCGGCG GCAGGCTGCAG  
301 GAGGAGGTGG AGGAGGTGAA GGCTCGGCCTC CAGGCCCTACA TGGCAGAGGC  
351 GCACCGAGCTG GTGGGCTGGAA ATTTGGAGGG CTTGCGGCAG CAACTGAAGC  
401 CCTACACCGAT GGATCTGATG GAGCAGGTGG CCCTGCCCGT GCAGGGAGCTG  
451 CAGGAGCAGT TGGCGGTGGT GGGGAAAGAC ACCAAGGCC AGTTGGCTGGG  
501 GGGCGTGGAC GAGGCTTGGG CTTTGCTGCA GGGACTGCAG AGCCCGCGTGG

FIG. 6B - 1

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551 TGCACCCAC CGGCCGCTTC AAAGAGGCTCT TCCACCCATA CGCCGGAGGC  
601 CTGCGTGAGCG GCATCGGGCG CCACCGTGCAG GAGGCTGCACC GCAGTGTGGC  
651 TCCGGCACGCC CCCGCCAGGCC CCGGGCGCCT CAGTCGCTGCG GTGCAGGGTGC  
701 TCTCCGGAA GCTCACGCTC AAGGCCAAGG CCCTGCACCGC ACGCATCCAG  
751 CAGAACCTGG ACCAGCTGCG CGAACAGGCTC AGCAGAGGCCT TTGCAGGGCAC  
801 TGGGAACTGAG GAAGGGGCCG GCCCGGACCC CCAGATGCTC TCCGAGGGAGG  
851 TGGGCCAGGG ACTTCAGGGCT TTCCGGCCAGG ACACCTACCT GCAGATAAGCT  
901 GCCTTCACTC GCGCCATCGA CCAGGGAGACT GAGGAGGTCC AGCAGCAGCT  
951 GGCCGCACCT CCACCGGCC ACAGTGCCCTT CGCCCCAGAG TTTCAACAAA  
1001 CAGACAGTGG CAAGGTTCTG AGCAAGGCTGC AGGCCCGTCT GGATGACCTG  
1051 TGGGAAGACA TCACTCACAG CCTTCATGAC CAGGGCCACA GCCATCTGGG

FIG. 6B-2

1101 GGACCCCTGA GGATCTACCT GCCCAGGCC ATTCCAGCT CCTTGTCTGG  
1151 GGAGCCTTGG CTCTGAGCCT CTAGCATGGT TCAGTCCTTG AAAGTGGCCT  
1201 GTTGGTGGA GGGTGGAAAGG TCCTGTGCAG GACAGGGAGG CCACCAAAGG  
1251 GGCTGCTGTC TCCTGCATAT CCAGCCTCCT GCGACTCCCC AATCTGGATG  
1301 CATTACATTC ACCAGGCTTT GCAAACCCAG CCTCCCAAGTG CTCATTGGG  
1351 AATGCTCATG AGTTACTCCA TTCAGGGTG AGGGAGTAGG GAGGGAGAGG  
1401 CACCATGCAT GTGGGTGATT ATCTGCAAGC CTGTTGCCG TGATGCTGGAA  
1451 AGCCTGTCGCC ACTACATCCT GGAGTTTGGC TCTAGTCACT TCTGGCTGCC  
1501 TGGTGGCAC TGCTACAGCT GGTCACAGA GAGGAGCACT TGTCTCCCCA  
1551 GGGCTGCCAT GGCAGCTATC AGGGAAATAG AAGGGAGAAA GAGAATATCA  
1601 TGGGGAGAAC ATGTGATGGT GTGTGAATAT CCCTGCTGGC TCTGATGCTG

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FIG. 6B - 3

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1651	GTGGGTACGA	AAGGTGTGGG	CTGTGATAGG	AGAGGGCAGA	GCCCCATGTTT
1701	CCTGACATAG	CTCTACACCT	AAATAAGGGA	CTGAAACCCTC	CCAAACTGTGG
1751	GAGGCTCCTA	AACCCTCTGG	GGAGCATACT	GTGTGCTCTC	CCCATCTCCA
1801	GCCCCCTCCCT	CTGGGGTTCCC	AAGTTGAAGC	CTAGACTTCT	GGCTCAAATG
1851	AAATAGATGT	TTATGAT			

FIG. 6B-4

1 MAVLTWALA LLSAFSATQA RKGFWDYFSQ TSGDKGRVEQ IHQQKMAREP  
51 ATLKDSLEQD LNMMNKFLEK LRPLSGSEAP RLPQDPVGMR RQLQEELEEV  
101 KARLQPYMAE AHELVGWNLE GLRQQQLKPYT MDLMEQVALR VQELQEQLRV  
151 VGEDTKAQLL GGVDEAWALL QGLQSRVVHH TGRFKELFHP YAESLIVSGIG  
201 RHVQELHRSV APHAPASPAR LSRCVQVLSR KLTLKAKALH ARIQQNLDQL  
251 REELSRAFAG TGTEEGAGPD PQMLSEEVRQ RLQAFRQDTY LQIAAFTRAI  
301 DQETEEVQQQ IAPPPIPCHSA FAPEFOQTDS GKVLSKLQAR LDDLWEDITH  
351 SLHDQGHSHL GDP\*

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FIG. 7

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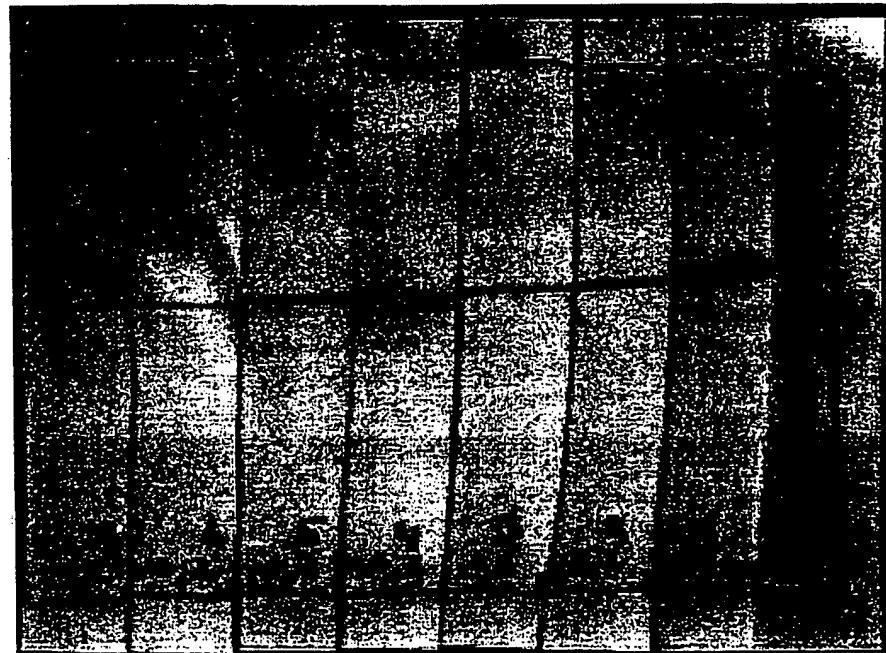


FIG. 8

3h                    6h                    12h  
hpx sham            pos.                hpx sham  
                      hpx sham            contr. hpx sham

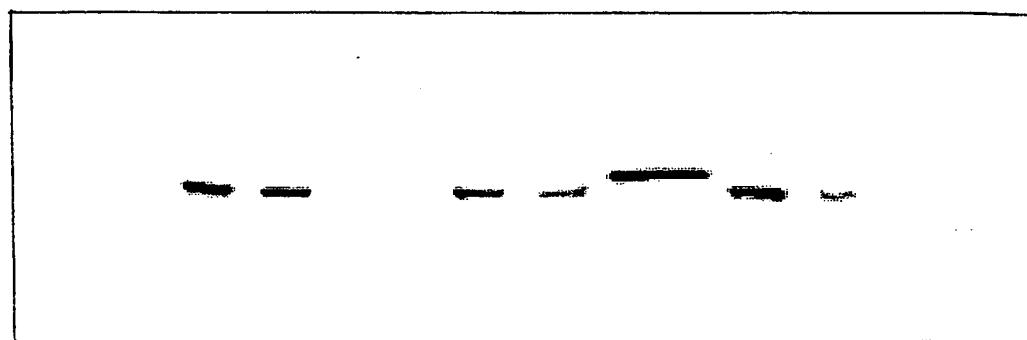


FIG. 9